

The University of Chicago

A STUDY OF THE EARLY EFFECTS OF
ANDROGENIC SUBSTANCES IN THE
RAT BY THE AID OF COLCHICINE

A PART OF A DISSERTATION SUBMITTED TO THE FACULTY OF THE
DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOOLOGY

1940

By

ELIZABETH Z. BURKHART

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A STUDY OF THE EARLY EFFECTS OF ANDROGENIC SUBSTANCES IN THE RAT BY THE AID OF COLCHINE ¹

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THREE TEXT FIGURES AND TWO PLATES (ELEVEN FIGURES)

INTRODUCTION

The first dependable short-time indicator for the endocrine secretion of the testis, the "spermatozoon motility test", was discovered and elaborated by Moore ('27, '28). Since that time a detailed study of the effects of male hormone in mammals has been in continuous progress in this laboratory and the first test has been followed by several other methods for studying the biological reactions of the hormones (for general discussion see Moore, '39). These methods require the restoration of cytological phenomena and an increase in gross weights of the accessory sex glands, or the production of secretions, or psychological responses in the castrated male mammal which approach or simulate those of the normal adult. The criteria are, in all cases, dependent upon repeated administration of the hormone. The hormonal effects are known to be rapid, but no detailed study of cell and tissue reactions has as yet been made to determine the earliest detectable responses and later gradual changes by which the definitive somatic characteristics evolve. Many fundamental questions regarding the nature of these biological reactions are yet to be settled; how the hormone exerts its stimulative effect is still unknown.

¹ This investigation has been aided by a grant from the Dr. Wallace C. and Clara A. Abbott Fund of The University of Chicago. It is a pleasure to acknowledge the services to this investigation of Dr. Gregory Stragnell, Schering Corporation, who has supplied the hormone preparations which were utilized.

The work of Allen and his co-workers (Allen and Creadick, '37; Allen, Smith and Gardner, '37; Rogers and Allen, '37) indicated that the peculiar action of colchicine on mitotic cells facilitates the detection of early responses in the female genital tissues following hormonal or other specific stimuli. Martins ('37) was the first to show that colchicine can be used to advantage in studying the mitotic activity in the ventral prostate of castrated mice and rats after treatment with androsterone and testosterone; he suggested its use in a rapid test for male hormones. Bastenie and Zylberszac corroborated these findings in the seminal vesicles of castrated prepubertal rats ('37 a) and castrated adult rats ('37 b) following three daily doses of testosterone propionate.

The results of a preliminary investigation on the application of colchicine to a study of the mitotic activity in androgen-stimulated accessory sex glands confirmed its advantage in such study (Burkhart, '39). It suggested a further examination of the ventral prostate and the seminal vesicles of castrated rats to determine (1) the progressive changes in the castrate accessory glands following the injection of a single dose of an androgen; (2) departures from that condition in response to varying amounts of androgen and after different periods of castration; and, (3) the specificity of mitotic activity as an indicator of testicular hormones.

This problem was suggested to me by Prof. Carl R. Moore. I am most grateful for his kindness in offering suggestions and criticism throughout the experimental work and the preparation of the manuscript. I wish to express my appreciation for the statistical analyses and suggestions for graphic representation of the data which were so kindly given by Prof. Sewall Wright.

MATERIALS AND METHODS

Over 500 male rats from an albino stock, which has been randomly bred in this laboratory, were used. All castrated rats were gonadectomized on day 40. Castration was ac-

completed under ether anaesthesia through a small mid-ventral abdominal incision. The length of time intervening between castration and treatment with hormones varied in different experiments as will be indicated below.

The stimulative effects of three synthetic hormones, viz., testosterone propionate (Oreton) and estradiol benzoate (Progynon-B) in oil and crystalline progesterone were studied. Except where deviations are indicated, the testosterone propionate solution contained 1 mg. per cubic centimeter.

Colchicine² (Eimer and Amend, amorphous) was dissolved in distilled water to contain 1 mg. per cubic centimeter. Fresh solutions were prepared frequently and were protected from light to prevent deterioration (Lits, '36). Following an exploratory study on the mitotic cells in the accessory glands in 27- and 66-day-old rats, a colchicine dosage of 0.1 mg. per 100 gm. body weight of rat was chosen. The proportionate amount was administered subcutaneously from a 1 cc. hypodermic syringe which was graduated to 0.01 cc.

A preliminary investigation (Burkhart, '39) showed that a maximal number of colchicinic mitoses accumulated in 6 hours; therefore this period of time was allowed to elapse between the injection of colchicine and sacrifice of the animals.

For autopsy the animals were lightly anaesthetized with ether, their body weights were recorded, and they were killed by decapitation. The ventral prostate, the seminal vesicles, and the coagulating glands (see fig. 26, Moore, '39) were removed with the aid of a low power ($\times 7.0$) binocular microscope. The glands were weighed on a torsion balance, and were immediately fixed in Bouin's fluid. Each autopsy required about 10 minutes.

The tissues were dehydrated in alcohol and methyl salicylate and embedded in paraffin. The ventral prostate and seminal vesicles were cut at 7 micra and were stained in Harris's and

² A survey of the literature on the history and nature of colchicine and its role in mitotic arrest has been presented in an earlier paper (Burkhart, '40).

Erlich's hematoxylin respectively according to Moore, Price and Gallagher ('30) and Moore, Hughes and Gallagher ('30).

Normal mitotic figures and colchicine-arrested mitoses were counted by means of an ocular micrometer disc with a field divided into forty-nine squares. Only the cells of the glandular epithelium were considered. The mitotic activity is recorded as the mean number of mitotic cells (exclusive of prophase nuclei) or of colchicine mitoses in twenty-five fields at a magnification of 570 diameters. The occurrence of only one, two, etc. mitotic cells in twenty-five, or more, fields is designated by 0.00 (1), 0.00 (2), etc.

EXPERIMENTAL RESULTS

A. Effects of 0.1 mg. of testosterone propionate

In preliminary experiments, castrated rats were given repeated daily doses of androgen; after colchicine treatment, they were sacrificed at daily intervals beginning on the second day. A few mitoses were present in the accessory glands of some animals at 31 hours after the first injection and thereafter the dividing cells were abundant up to 5 days after the first injection.

Two rats which had been given five daily doses of 0.1 mg. of testosterone propionate, one of which had also received colchicine, were killed on the sixth day. The accessory glands of both rats showed fewer mitoses than did any animal killed from the second to the fifth day. No secretion granules had appeared in the seminal vesicles of either of these two animals.

The acinous diameters of the ventral prostates were much increased over those of the castrated control; the glandular cells were large and swollen and, along the periphery of the gland, pale light areas appeared. The histology of the glands and the gross weights indicated marked growth changes.

Despite the need for further study of the changes in the accessory reproductive glands over a longer period of time, it appeared that the first step in understanding the effects of synthetic androgens should be concerned with the determina-

tion of (1) the speed of effectiveness (2) the time of maximum effect, and (3) the duration of effectiveness.

Since the results in the preliminary experiments were complicated by repeated doses of androgen, single doses of 0.1 mg. of testosterone propionate were injected into 40-day castrated rats on their eightieth day of age. Starting at 15 hours after the administration of the androgen the animals were sacrificed at 4-hour intervals up to 67 hours and thereafter at 8-hour intervals to 115 hours, with a last group at 126 hours. Some of the rats were colchicine-treated while others served as "controls" without colchicine. In addition, nineteen colchicine-treated castrated animals which had not received androgenic treatment were studied as "castrate controls" (see table 1).

TABLE 1

Averaged data from "castrate control" rats which had not received testosterone propionate.

Number of rats	19
Mean age at castration (days)	39.6 $\pm$ 0.02
Mean age at autopsy (days)	81.8 $\pm$ 1.07
Mean body weight (gm.)	174.6 $\pm$ 5.01
Mean gland weights (mg.)	
Seminal vesicle	9.9 $\pm$ 0.47
Ventral prostate	8.3 $\pm$ 0.33

The gross weights of the hormone-treated glands (table 2 and fig. 1) are seen to be very variable. Most of them, however, fall well above the mean of the nineteen weights from the "castrate control" animals. Prof. Sewall Wright has statistically analysed the data on the weights of the ventral prostates and the seminal vesicles from 67 to 126 hours and finds a significant trend of decreasing weight in the ventral prostate. Failure to show the same in the seminal vesicles is probably due to the small number of animals; histologically they exhibit the same gradual atrophy as the ventral prostates.

The earliest histologically perceptible effect of treatment appears in the glandular epithelium at 23 hours (the castrate

TABLE 2

Response of the accessory glands of 80-day-old rats which were castrated on day 40 and injected with 0.1 mg. of testosterone propionate.

TIME IN HOURS AFTER TESTOSTERONE PROPIONATE	CONTROL					COLCHICINE-TREATED				
	No. of animals	Averaged weights of accessory glands in mg.		Averaged mean numbers of normal mitoses per field		No. of animals	Averaged weights of accessory glands in mg.		Averaged mean numbers of colchicine mitoses per field	
		<i>s. v.</i>	<i>v. pr.</i>	<i>s. v.</i>	<i>v. pr.</i>		<i>s. v.</i>	<i>v. pr.</i>	<i>s. v.</i>	<i>v. pr.</i>
15	0					1	13.8	8.5	0	0.02
19	1	19.0	8.0	0	0	1	10.0	7.4	0	0.02
23	2	15.0	10.8	0.01	0.01	2	20.05	11.1	0.06	0.03
27	2	12.9	9.0	0.02	0.01	2	15.6	10.4	0.35	0.07
31	5	16.8	10.5	0.65	0.41	4	14.5	10.6	0.50	0.20
35	4	17.7	12.1	0.71	0.53	6	14.4	11.0	3.79	2.21
39	3	14.6	9.4	3.36	2.22	6	14.0	11.6	16.78	12.29
43	2	15.5	12.9	6.33	5.72	3	16.3	13.2	96.24	30.28
47	2	17.5	14.6	7.56	3.40	3	16.8	13.3	75.92	52.82
51	2	18.9	14.4	1.08	1.13	4	17.7	13.4	79.91	36.37
55	1	14.4	13.8	0.20	0.94	3	25.9	17.8	67.76	22.80
59	1	17.7	14.0	4.60	2.01	3	23.5	17.7	53.49	20.28
63	1	16.2	13.8	0.44	0.42	4	15.7	13.5	27.16	8.54
67	1	19.2	16.8	0.78	0.66	3	22.7	16.5	25.86	10.84
75	1	14.4	12.5	0.32	0.04	3	16.1	11.8	8.14	6.30
83	1	15.5	12.8	0.56	0.18	3	20.2	14.4	4.86	2.35
91	1	19.4	12.4	0.40	0.62	3	17.1	12.9	0.73	1.48
99	1	13.0	8.8	0.04	0.02	1	28.3	18.3	2.16	6.54
107	0					2	15.3	12.5	0.34	0.28
115	0					4	10.8	10.0	0.10	0.16
126	1	14.0	9.0	0	0	2	15.1	12.6	0	0

condition is illustrated by figs. 4, 8 and 11). Stimulation manifests itself by an increase in the amount of cytoplasm and in the size of the nuclei. In the seminal vesicle the elongate nuclei become more oval. The thin film of cytoplasm along the luminal side of the cells widens and takes a heavier stain.

Fig. 1 Distribution of the glandular weights of the ventral prostate and the seminal vesicle (including both the control and the colchicine-treated animals) at intervals following the injection of 0.1 mg. of testosterone propionate. The dotted line (.....) joins the mean weights at each interval. The solid line (—) indicates the mean weight of the respective glands from the nineteen "castrate control" animals of similar age and castration history (table 1).

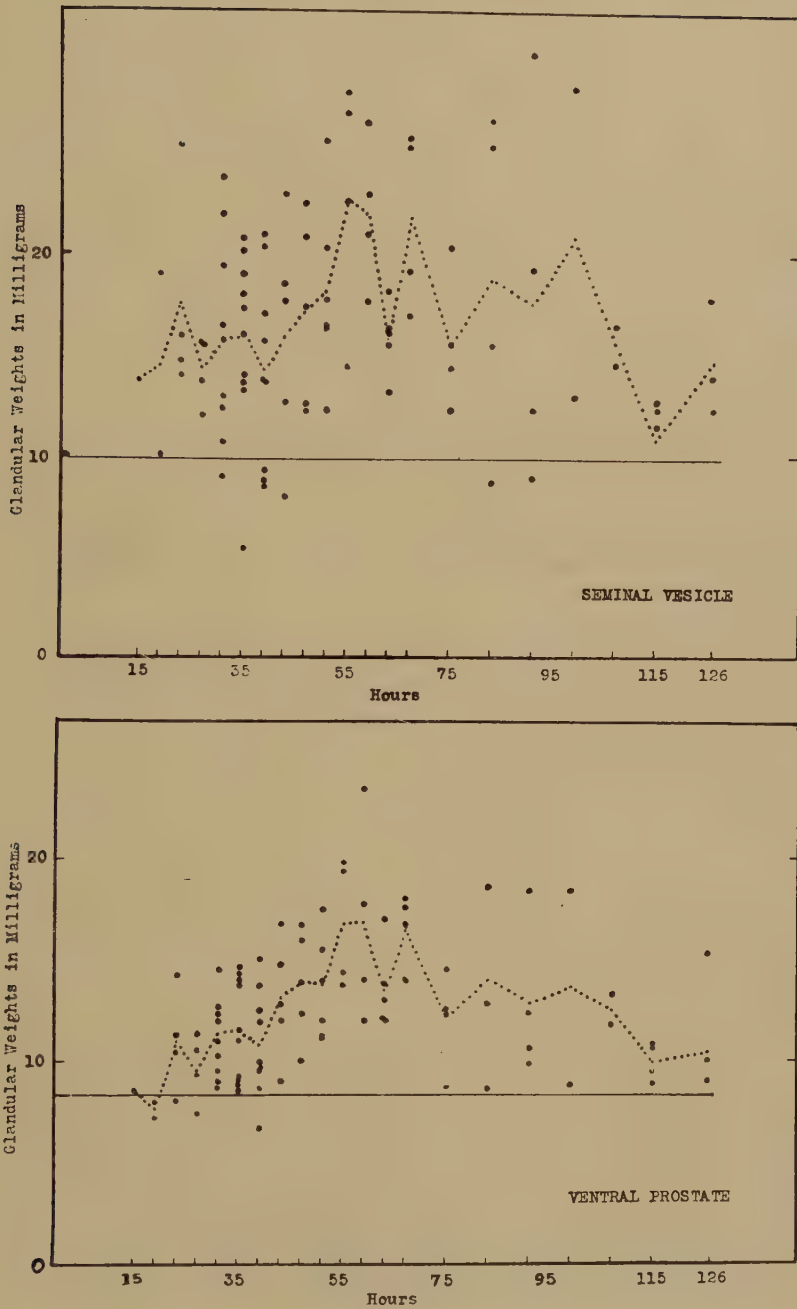


Figure 1

The villi which project into the ventral lumen and into the lumina of the lateral branches of the gland, finally fill these spaces. In the ventral prostate, irregular or oval nuclei become more coarsely granular; the cellular volume increases causing the acini of the gland to expand. This hypertrophy continues and is followed at the thirty-fifth hour by cell division. Changes in the glands do not proceed at equal rates throughout the whole gland: both hypertrophy and mitosis start earlier at the apices of the lateral branches in the seminal vesicle; in the ventral prostate they are first apparent along the periphery of the gland.

The changes in the fibromuscular connective tissue of the seminal vesicle and in the loose connective tissue of the ventral prostate are more difficult to detect and describe. In both glands these tissues hypertrophy and as the glandular epithelial elements grow, they are stretched, in the seminal vesicle, or crowded, in the ventral prostate, by the expansion. Mitoses do occur in the connective tissue of both glands, but, even at the height of mitotic activity in the glandular cells, they are very infrequent. In the glandular epithelium significant mitotic activity starts at about 35 hours; it becomes more intense during the 43- to 59-hour period after which it declines. In the graph (fig. 2) it is shown that the number of mitoses rises at the same time in the glands of both the colchicine-treated and the control animals. Mitoses in the control glands are comparatively few in number because they are only those cells which were undergoing mitosis at the moment of death. The data indicate that a great number of cells become mitotic at the same time.

Since the gland of the colchicine-treated animal (fig. 7) and its control (fig. 6) show similar hypertrophic changes and mitotic activity at the same time after treatment with testosterone propionate, these phenomena are not caused by the colchicine. It is assumed, therefore, that colchicine merely arrests the mitotic cells which would have divided during the 6-hour period preceding death. (Compare figs. 6 and 7 with the colchicine-treated castrate condition in fig. 8).

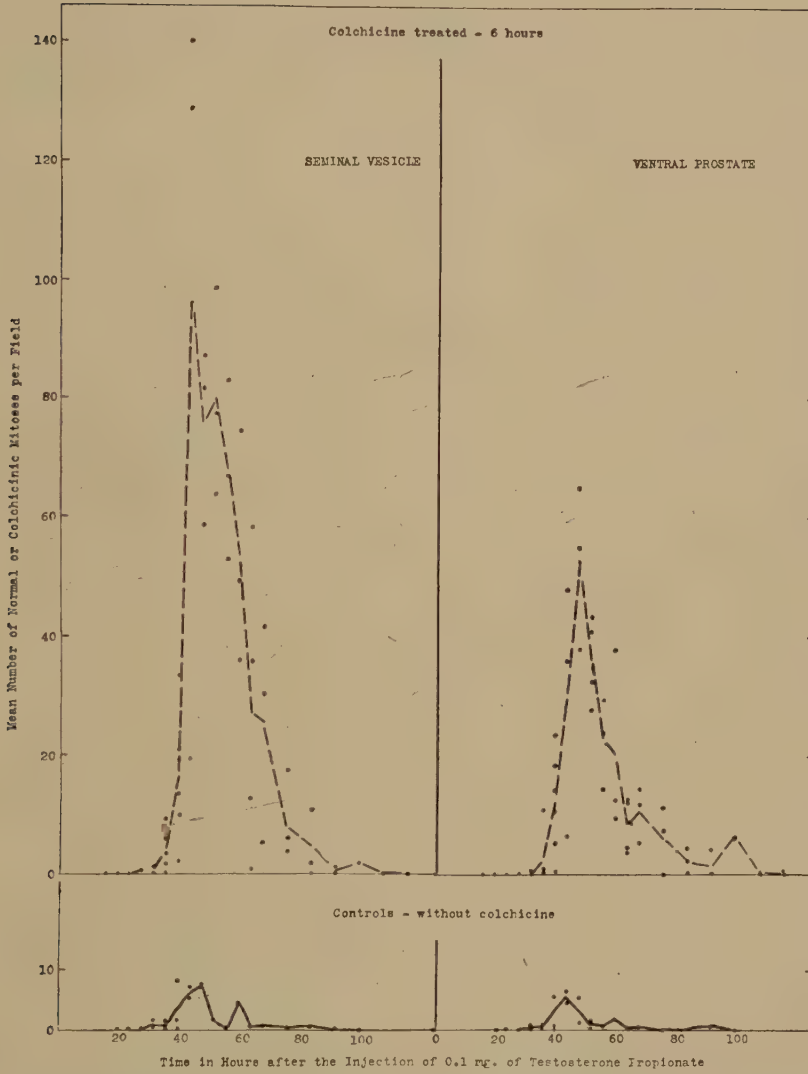


Fig. 2 Distribution of the mean numbers of colchicine figures (above) and of normal mitoses (below) at intervals after the injection of 0.1 mg. of testosterone propionate. The broken line (— — — —) is drawn through the mean of means at each interval. The solid line (————) represents the same for the animals which did not receive treatment with colchicine.

There is no significant difference between the time of response of the seminal vesicle and the ventral prostate. The average number of mitotic figures is greater for the seminal vesicle because the epithelium is more compact and the cells are smaller (compare figs. 4 and 8; 5 and 7).

The occurrence of a mitotic or colchicine cell in a "castrate control" is very infrequent.

Variability in the gross weights of the glands and in the mitotic activity in different animals, at the same time interval, is great; it may arise from several sources. In the first place, it was impossible to use one group of litter mates for each time period because of the time required for autopsy; the colchicine period was kept as strictly as possible to 6 hours. The administration of the androgen was also subject to inaccuracies; when this problem was begun the testosterone propionate was injected subcutaneously from a 2-cc. syringe graduated to 0.05 cc. After the reaction was found to be so intense it was injected from a 1-cc. syringe graduated to 0.01 cc. Undoubtedly, individual physiological differences also account for some variation.

B. Effects of 0.3 mg. and 0.05 mg. of testosterone propionate

The preceding study clearly delimited the preferable time for observing maximal effects of hormonal stimulation. It also suggested the use of larger and smaller doses.

One series of twelve rats and a second of sixteen rats, castrated on day 40, were injected on the eightieth day with 0.05 and 0.3 mg. of testosterone propionate respectively. They were sacrificed in pairs at 4-hour intervals up to 48 hours. One-half of the animals in each group was treated with colchicine; the other half was not. The results are recorded in table 3. A comparison of these data with those of the animals which received 0.1 mg. of androgen is made by a graph (fig. 3).

The smallest amount of androgen injected (0.05 mg. in 0.05 cc. of oil) is sufficient to elicit a variable response in both glands. The largest dose (0.3 in 0.3 cc. of oil) causes an

"explosion" of mitoses at 35 hours in both the control and the colchicine-treated animals. The numbers of mitoses in the control glands at 27 and 30 hours suggests that the latent period might be found to be shorter in a larger sample of animals. Certainly, the latent period which precedes mitotic

TABLE 3

Mitotic activity in 80-day-old rats, castrated 40 days and treated with 0.05 and 0.3 mg. of testosterone propionate.

TIME IN HOURS AFTER TEST. PROP.	0.05 MG. OF TEST. PROP.				0.3 MG. OF TEST. PROP.			
	Control		Colchicine- treated		Control		Colchicine- treated	
	Mean number of mitotic cells per field		Mean number of colchicine mitoses per field		Mean number of mitotic cells per field		Mean number of colchicine mitoses per field	
	s. v.	v. pr.	s. v.	v. pr.	s. v.	v. pr.	s. v.	v. pr.
15.....					0	0	0.04	0.04
19.....					0	0	0.24	0.16
23.....	0.04	0.08	0.44	0.28	0.28	0	0.20	0.40
27.....	0	0.02	1.68	0.64	5.00	0.76	2.04	0.56
31.....	2.60	0.04	2.64	0.82	2.92	0.26	7.60	0.10
35.....	4.00	0.36	0	0	10.68	7.80	108.60	29.40
39.....	1.36	0.18	5.32	0.44	7.92	7.86	89.50	37.66
43.....	1.44	0.32	6.36	22.52	6.92	3.76	107.50	61.10
Mean body weight of 12 rats: 169 $\pm$ 4.98 gm.					Mean body weight of 16 rats: 175 $\pm$ 4.77 gm.			

activity in response to 0.3 mg. of androgen is shorter (at least 8 hours) than that following the administration of 0.1 mg. of testosterone propionate.

C. The minimal reactive dose of testosterone propionate

The discovery that a single dose of 0.05 mg. of testosterone propionate was sufficient to induce mitotic activity in the castrate glands and the suspicion that at low dosages the ventral prostate was relatively more reactive than the seminal vesicles, led to an interest in determining the minimal effective dose.

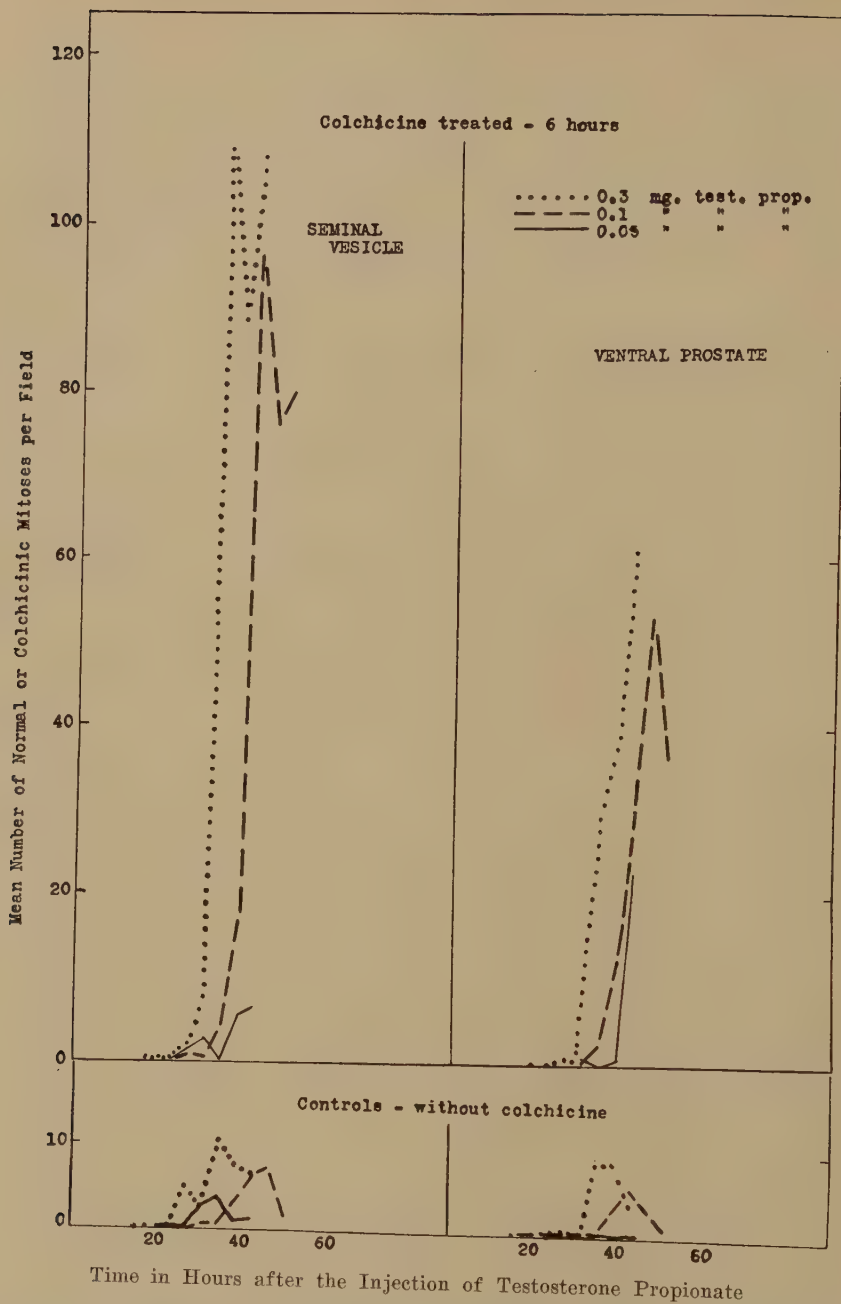


Fig. 3 Mean numbers of colchicine mitoses (above) and of normal mitoses (below) following the injection of varying doses of testosterone propionate. (Data from tables 2 and 3).

Therefore, single graded doses, ranging from 0.05 mg. to 0.006 mg. of androgen were injected into rats of the same age and castration history as those in the preceding experiments.

The preparation containing 1 mg. of testosterone propionate per cubic centimeter of oil was diluted with sesame oil so that 1 cc. of oil contained 0.1 mg. of testosterone propionate. Four litters of castrated animals were injected with 0.05, 0.025, 0.013 and 0.006 mg. of the hormone respectively. One control animal in each litter received an equivalent amount of sesame oil. A fifth group received 0.3 cc. of testosterone propionate diluted to contain 0.01 mg. of hormone per cubic centimeter. Some of the animals were colchicine-treated while others were not (see table 4). They were killed at a time when, according to the reaction with 0.1 mg., mitotic activity could be expected.

The response of the glands to these small doses was extremely variable and the seminal vesicle was hardly affected by any of them. It is to be remembered that any mitotic count less than that of the prostate (see above, p. 144) is significantly smaller as a measure of the stimulated activity. It will be noted that dividing cells occur occasionally in colchicine-treated "castrate controls". The few cells found, therefore, in the androgen treated glands in group IV and group V have no significance as indicators of positive stimulation. This conclusion is supported by the absence of hypertrophy which always precedes and accompanies mitosis.

It is interesting that the seminal vesicle responded to treatment with 0.05 mg. of androgen when it was injected in 0.05 cc. of oil but showed a very feeble response when the same amount was administered in 0.5 cc. of oil. The dilution of the androgen may play some role in the intensity of the mitotic activity.

Mitosis in the ventral prostate after treatment with 0.013 mg. of testosterone propionate (fig. 9) is further evidence that the ventral prostate has a lower threshold of response than the seminal vesicle (Moore and Gallagher, '31; Moore and Price, '37, '38).

It follows, therefore, that 0.013 mg. is the lowest single injected dose that yields a positive response in the ventral prostate but it requires more than 0.05 mg. to give a comparable response in the seminal vesicle.

TABLE 4
Mitotic activity in 80-day-old rats, castrated 40 days and treated with minute doses of testosterone propionate.

TIME IN HOURS ¹ AFTER TEST. PROP.	CONTROL			COLCHICINE TREATED		
	Rat. No.	Mean No. of Mitotic Cells per field		Rat. No.	Mean No. of Colchicine Mitoses per field	
		s. v.	v. pr.		s. v.	v. pr.
Group I. 0.05 mg. Testosterone Propionate						
45.....	464	0.04	0	466	0	0.16
45.....	465	0	0.32	467	0.16	2.68
45.....				468	3.88	9.72
0.....				463	0	0
Group II. 0.025 mg. Testosterone Propionate						
46.....	470	0	0.56	472	0.00(2)	0.36
46.....	471	0	0.56	473	0	0
46.....				474	0.00(1)	0.48
0.....				469	0	0
Group III. 0.013 mg. Testosterone Propionate						
46.....	476	0	0.12	478	0.00(2)	0.33
46.....	477	0	0.20	479	0.21	3.44
46.....				480	0.45	6.88
0.....				475	0	0.00(3)
Group IV. 0.006 mg. Testosterone Propionate						
47.....	482	0	0.04	485	0.00(2)	0.32
47.....	483	0	0.08	486	0	0
0.....				481	0	0
Group V. 0.003 mg. Testosterone Propionate						
47.....	488	0	0	490	0	0.12
47.....	489	0	0	492	0	0
0.....				487	0	0

¹The designated time is approximate; the animals were killed successively over a period of 3 hours starting at 45 hours.

D. Influence of the duration of castration on the response to 0.1 mg. of testosterone propionate

When this problem was begun, the 40-day period of castration was rather arbitrarily selected to assure complete regression of the accessory sex glands and thereby to reduce the possibility of spontaneous mitosis. The need for this long-extended period had not been indicated by experimental evidence. It was of interest to learn (1) whether or not this precaution is necessary and (2) whether the duration of castration modifies the response of the glands.

Forty-two rats were castrated at 40 days of age. Eighteen of them received 0.1 mg. of testosterone propionate 10 days after they were castrated; two animals without androgen served as "castrate controls". Nineteen animals received 0.1 mg. of testosterone propionate 20 days after castration; three 60-day-old rats were sacrificed as "castrate controls". The androgen treated rats were killed in pairs (one control and one colchicine-treated) at 4-hour intervals.

The results of this experiment are recorded in table 5. The untreated "castrate controls" are omitted from the table; no mitotic or colchicine cells were observed either at 10 days or at 20 days after castration. Large numbers of mitotic cells appear in the seminal vesicles of the 50- and 60-day-old rats at 39 hours. In the ventral prostate mitosis lags until about 47 hours. Since each time interval is represented by only one pair of rats the data are very limited. However, the results are quite consistent and indicate a difference in response of the two glands shortly after castration. It is rather surprising to find delayed mitosis in the ventral prostate because at 40 days after castration it responds to lower dosages than the seminal vesicle. It has also been shown that, during the prepubertal period, functional differentiation is evident much earlier in the ventral prostate than in the seminal vesicle (Price, '36). The delayed mitotic response of the ventral prostate may be associated with the slower dedifferentiation following castration (Moore, Hughes and Gallagher, '30;

Moore, Price and Gallagher, '30). The difference in behavior of the two glands shortly after castration would be of importance if these techniques were to be applied to a quantitative test for hormones.

TABLE 5

Mitotic activity in 50- and 60-day-old rats castrated on day 40 and treated with 0.1 mg. of testosterone propionate.

TIMES IN HOURS AFTER TEST. PROP.	50-DAY-OLD				60-DAY-OLD			
	Control		Colchicine-treated		Control		Colchicine-treated	
	Mean number of mitotic cells per field		Mean number of colchicine mitoses per field		Mean number of mitotic cells per field		Mean number of colchicine mitoses per field	
	s. v.	v. pr.	s. v.	v. pr.	s. v.	v. pr.	s. v.	v. pr.
19.....	0.08	0	0.08	0	0	0	0	0.04
23.....	0.52	0	0	0	0	0	1.08	0.04
27.....	0	0	0.76	0	0.16	0.04	0.64	0.08
31.....	0.12	0	0.08	0	0	0	4.40	0.12
35.....	0.16	0	0.44	0			3.12	0.04
39.....	2.28	0.32	57.32	4.56	1.08	0.14	20.24	1.48
43.....	2.76	0.28	60.70	8.20	6.76	0.76	55.10	0.76
47.....	3.12	0.72	0.72	1.00		1.92	81.60	29.20
51.....	6.00	1.12	64.60	53.88		0.20	99.40	25.80
55.....					3.80	3.76	80.20	28.10
Mean weight of 20 rats: 110.6 $\pm$ 9.18 gm.					Mean weight of 22 rats: 128 $\pm$ 3.53 gm.			

E. The stimulative capacity of other synthetic hormones

1. *Progesterone.* Progesterone, presumably never present in a male rat, has been shown by Lamar ('37, '40) and by Greene, Burrill, and Ivy ('39) to exert some stimulation on the accessory reproductive glands of castrated males. This "androgenic" effect appears to be limited to the ventral prostate in which Lamar has noted that, in response to repeated large daily doses, light areas were developed. However, he was unable to cause the development of secretion granules in the seminal vesicle and suggested, without definite proof, that it was because the dose was insufficient. Since mitotic activity has

been shown to be an adequate test for determining very slight difference in the response of these glands, it became of interest to learn whether the lack of secretory differentiation in the seminal vesicle is, indeed, due to quantitative factors or whether the substance evokes a response which is qualitatively different. By the following procedure very large doses of progesterone were injected into males castrated for 40 days.

Crystalline progesterone (Schering) was dissolved in sesame oil by gentle heating on the outer metal strip of a paraffin oven. The solution contained 20 mg. of dissolved progesterone per cubic centimeter. Four out of a litter of five castrated rats were injected with 1 cc. on the eightieth day of age. One day later three of the rats received a second 20-mg. dose. All of them were treated with colchicine and all of them were killed at about 56 hours after the first injection of progesterone.

TABLE 6

Mitotic activity in the accessory glands of rats castrated at 40 days and injected with progesterone at 80 days of age.

RAT NO.	BODY WEIGHT IN GM.	TOTAL DOSE OF PROGESTERONE IN MG.	ACCESSORY GLAND WEIGHTS IN MG.		MEAN NO. OF COLCHINIC MITOSES PER FIELD	
			<i>s. v.</i>	<i>v. pr.</i>	<i>s. v.</i>	<i>v. pr.</i>
541	188	none	13.7	12.0	0	0
542	196	20	11.2	8.9	0.28	0.48
543	182	40	12.0	9.5	0.08	2.72
544	219	40	14.3	13.8	1.48	8.32
545	184	40	13.9	11.9	0.12	0.12

A very few mitoses were found in both glands after treatment with only one 20-mg. dose of progesterone (rat no. 542), and they were more numerous than those in rat no. 545 which had received a second dose of the hormone. The greatest stimulation was elicited in rat no. 544 (see figs. 10 and 13 and compare them with figs. 11 and 12). Hypertrophy is pronounced in the glandular epithelium and connective tissue of both glands. The ventral prostate contains actually and pro-

portionately more colchicine cells and is apparently more sensitive to progesterone than the seminal vesicle. The marked hypertrophy, particularly in the fibromuscular connective tissue of the seminal vesicle, coupled with the feeble mitotic activity, connotes a qualitatively different action of the progesterone from that of testosterone propionate.

2. *Estradiol benzoate*. Numerous authors have described the stimulation of the accessory glands of normal and castrated male rodents, dogs, and monkeys which follows the repeated administration of large doses of estrogenic substances (Allen, Hisaw, and Gardner, '39; Wells, '36). Species differences in the response of the glands are pronounced. The seminal vesicles of castrated mice were atrophied after 5 months treatment (Lacassagne, '33). Mitotic cells occurred in the glandular epithelium and in the connective tissue of castrated mice a week after the injection of a total dose of 200 gamma of estradiol benzoate (Tislowitz, '39 a). The ventral prostate became enlarged due to hypertrophy of the connective tissue and the accumulation of fluid in the lumina of the tubules (Lacassagne, '33). Others have described extreme metaplasia of the glandular epithelium resulting in a stratification and cornification simulating the histological condition of an estrous vagina (Weller, Overholser, and Nelson, '36; Lacassagne et Villela, '33; Burrows, '35; Burrows and Kennaway, '34; Tislowitz, '39 a). The accessory glands of the ground squirrel respond to estrogenic treatment in the latter manner. Rats behave quite differently. The involution of the seminal vesicles and the ventral prostates of castrated rats was not prevented by treatment with oestrin (Moore and Price, '32) but the fibrous tissues of the seminal vesicle increased markedly after prolonged injection (Freud, '33; Overholser and Nelson, '34, '35; Korenchevsky and Dennison, '35). The latter authors state that "the changes in the ventral and lateral lobes (of the prostate) were slight." Weller, Overholser and Nelson ('35) report a suppressing effect upon the epithelium of the prostate.

Because of the controversial aspects of these experiments it was desirable to study the preliminary changes in the accessory glands following a large single dose of the estrogen. One cubic centimeter of oil containing 2000 R. U. (Allen-Doisy) of estradiol benzoate was diluted with sesame oil to contain 100 R. U. per 1 cc. Eight 40-day castrated rats were injected with doses of 100 R. U. each. One colchicine-treated animal was sacrificed 27 hours after the injection of the estrogen and others were killed at 4-hour intervals up to 55 hours. A careful comparison of the ventral prostate and the seminal vesicles with their respective "castrate controls" reveals a striking difference in the response of the two. The ventral prostate is not different in any way or at any time from the control: not a single mitotic figure was found in the glandular epithelium and the connective tissue resembles the castrate in all details. The seminal vesicle, in contrast, is stimulated. At 27 hours, growth is evident in the nuclei and cells of the glandular epithelium. By 39 hours numbers of mitotic cells are present; they continue to be found in all the tissues up to 55 hours (fig. 14). Slight hypertrophy and an occasional mitotic cell is seen in the connective tissue (compare figs. 12 and 14). The seminal vesicle is responsive to a single large dose of estradiol benzoate.

DISCUSSION

The first action of an androgenic substance is to increase the cell and nuclear contents in the tissues which are sensitive to its presence. As its stimulation continues the cell is brought to a physiological state where cell division is inevitable. The stimulative action of a single dose of 0.1 mg. of testosterone propionate in a rat weighing approximately 170 gm. initiates these processes and continues them over a period of at least $2\frac{1}{2}$ days; after that the cells return to a castrate condition. When injections are repeated it is well known that the cells not only increase in size and number but they become differentiated for their special secretory functions. The amazing capacity of a single synthetic substance to induce all these

reactions in specifically sensitive tissues raises many questions of vital importance to all biological processes. The reactivation which takes place in the individual glandular cells of the accessory sex organs parallels in many respects the rejuvenation of "depressed" Protozoan cultures; in a favorable environment and in the presence of available food substances they increase in size, then undergo fission and finally become differentiated for vegetative functions. Many substances are known to be essential for the growth of living things: vitamins, tissue extracts, bios, auximones (Allee, '31), and numerous growth-promoting hormones in plants and animals. They are necessary in very small amounts and appear to have a "conditioning effect". Very little is known about the specific effects of any of these substances and the question is not answered by our present knowledge of hormonal action. Does the hormone supply a specific nutritive substance? That would hardly seem possible after observing the intense reaction and the changes which are caused by only 0.1 mg. of testosterone propionate. It may be that the androgen makes available to the glands substances which are present in the body at all times by specific action upon physiological mechanisms, i.e. vascular, nervous, or metabolic processes, which are essential to the normal activities of these glands.

The fact that the hormone produces responses more readily in localized areas in the glands, that is, along the periphery of the ventral prostate and in the projecting apices of the lateral branches of the seminal vesicle indicates an intimate relationship with the morphology of the gland as a whole.

These early effects of androgens focus attention upon the evolution and development of cell inclusions and upon their interrelationships with cell division and cell metabolism. What is the order of appearance of the cell structures characteristic of an actively secreting cell? Is it possible to administer the testosterone propionate in a quantity or in a milieu which would cause secretory differentiation without preliminary mitosis? In the seminal vesicle progesterone induces cellular enlargement with very little mitosis; Lamar has shown that

it is difficult, perhaps impossible, to induce the formation of secretion granules. In the ventral prostate, on the other hands, where mitoses do occur under the influence of progesterone the cells also show secretory differentiation after long-continued treatment. Estradiol benzoate stimulates mitosis in the seminal vesicle but has no detectable effect upon the ventral prostate. These reactions are apparently qualitatively different.

The period which intervenes between the first evidence of stimulation (beginning hypertrophy) at 23 hours and the onset of mitosis at about 35 hours indicates that the glandular cells of the ventral prostate and the seminal vesicle in a 40-day-castrated animal have regressed below the condition essential for mitotic activity. The cytological requirements for cell division are not well understood. It is known that compensatory hypertrophy (involving also hyperplasia) follows the removal of one of a pair of organs from the body. Brues and Marble ('37) describe a latent period of 24 hours before mitosis starts in regenerating liver tissue after partial hepatectomy. During the period the "organ grows rapidly" and shows none of the characteristics ordinarily attributed to undifferentiated tissues. They found also that explants of liver tissue taken from animals beyond the latter half of embryo life will not proliferate. The latent period, they add, was generally thought to be the time necessary for cells to become dedifferentiated. That hyperplasia can be induced by other stimuli than hormonal action, ablation, or injury is shown by the work of Allen, Smith, and Reynolds ('37). With the aid of colchicine treatment, they were able to detect mitotic activity in the uterine wall of an ovariectomized rabbit after 3 days distention by inserted pellets, in the absence of known hormonal stimuli. Until more experimental evidence has accrued, a discussion about the latent period and the nature of the cytological changes preceding cell division is mere speculation. It seems clear that mitotic cells, which, it is generally believed, require preliminary dedifferentiation from a functional state, must be specifically differentiated for

the process of division and that regression can proceed below that condition in the accessory glands of a rat that has been castrated for a long time.

Furthermore, the ventral prostate appears to attain the physiological condition essential for mitosis more slowly than the seminal vesicle following castration. In the seminal vesicle mitosis follows androgenic treatment at the same time in the 10-, 20- and 40-day castrated animal. In the ventral prostate mitosis is delayed about 8 hours in the 10- and 20-day castrated rat but occurs simultaneously with the seminal vesicle in a 40-day castrated animal.

The foregoing discussion is concerned with the intrinsic reactivity of the living tissues. The speed of the reaction of a gland may be in part determined by the state of the hormone, the method of administration, etc. Despite the sporadic appearance of a few mitoses before the maximum of activity, the first period of stimulation ends with maximal cell division and is known as the latent period.

When 0.1 mg. of testosterone propionate in 0.1 cc. of oil is injected, the latent period is about 43 hours; this period can be shortened by the injection of 0.3 mg. of testosterone propionate in 0.3 cc. of oil. The possibility that a larger amount of oil may be more rapidly distributed, thus facilitating more rapid absorption, must not be overlooked. Deansley and Parkes ('37) observed that testosterone propionate crystals, in pellets, are very slowly soluble in the body fluids and the hormone is therefore less easily absorbed than closely related substances like testosterone, which are more soluble. They believe that the slower rate of absorption increases the effectiveness of the androgen by simulating the slow, continuous secretion of the normal gland. The latent period following testosterone propionate is very much longer than that following the injection of theelin into spayed female mice. Allen, Smith and Gardner ('37) found "rapid growth in full swing" and mitoses in the genital tissues at $9\frac{1}{2}$ hours after the injection of 50 I. U. of theelin, in oil, into mice which had been ovariectomized 2 months previously. Tislowitz found the

latent period, following the administration of testosterone into infantile mice, which had been castrated 12 to 14 days, to be shorter than that following the injection of the same amount of testosterone propionate. The simultaneous injection of testosterone and Laqueur-X substance lengthened the latent period and produced greater mitotic activity, both of which are characteristics of the action of the propionate (Tislowitz, '39 b). Many substances are known to affect the potency of androgens, particularly in the rat (Gustavson, '39).

The experimental results of this problem indicate that, with the action of colchicine, it is possible to detect very slight differences in the response of castrated accessory glands to androgenic substances. Loewe and Voss ('30) were the first to suggest hyperplasia in the castrate seminal vesicle as a possible indicator for testicular hormone. The method did not gain acceptance and many other techniques were developed and have become standardized tests for androgens (Moore, '39; Koch, '39; Gustavson, '39).

The colchicine reaction in the detection of mitotic activity adequately satisfies the requirements for a biological assay of hormones (Moore, '39). Mitotic activity is the indicator upon which the assay depends. It embodies all the precursors of later changes in the gland, viz., growth increment, hyperplasia, and cell differentiation. Until a substance is discovered which produces a normal adult type of accessory gland in a castrated animal without mitotic activity, mitosis may be considered essential for further development and is as dependable as any later manifestations of stimulation. Mitotic activity in the glands, particularly in the ventral prostate, is clear and definite in response to single, very small doses of androgen.

This work suggests that colchicine-modified mitosis may be used as a quantitative as well as a qualitative test for hormones. The changes which follow the injection of testosterone propionate appear to be influenced by the amount and dilution of the androgen, and the length of the castration period. These factors alter the latent period, the intensity of mitosis, and,

probably, its duration. Further study is needed to determine these interrelationships.

Since the test is sensitive enough to detect very small amounts of hormone following one injection, it is subject to inaccuracies arising from the technique of administration. However, it should be no more subject to variations in the individual physiological and morphological differences of the test animals than the capon comb growth method or the quantitative tests based upon weight increment in the accessory glands of rodents.

In rapidity of application, it excels all other known tests. Only one application of the test substance is required and the responses can be determined within 2 or 3 days.

The mitotic activity in the accessory glands of male rats which follows the injection of synthetic female-like hormones, appears to invalidate it as an indicator of androgens. Close scrutiny of the data and of the tissues reveals very characteristic differences in the behavior of the three substances studied. (1) Very large doses of estradiol benzoate (100 R. U.) and of progesterone (40 mg.) elicit comparatively little mitotic activity. (2) At 55 hours after hormonal treatment, the estradiol benzoate causes mitosis in the seminal vesicle but none in the ventral prostate (see also Fleischmann and Kann, '38). (3) Progesterone produces more mitotic cells in the ventral prostate than it does in the seminal vesicle. Marked hypertrophy in the glandular cells and in the fibro-muscular connective tissue is also a characteristic of progesterone stimulation. (4) The intense mitotic activity in both the seminal vesicle and the ventral prostate following the injection of only 0.1 mg. of testosterone propionate is specific for this androgen.

SUMMARY

1. The first evidence of stimulation in the accessory sex glands of a 40-day castrated rat, following the injection of 0.1 mg. of testosterone propionate is slight hypertrophy; it begins at 23 hours and starts to decline at about 67 hours. At the end of 43 hours the height of mitotic activity is reached simul-

taneously in the seminal vesicles and in the ventral prostate. Cell division continues in decreasing numbers until it ceases at about 107 hours after the injection of the hormone. At 126 hours the glands resemble the castrated condition.

2. Three-tenths milligram of testosterone propionate in 0.3 cc. of oil shortens the latent period in 40-day castrated rats but does not significantly increase the initial intensity of mitosis.

3. Both the seminal vesicle and the ventral prostate show mitotic activity following the administration of one dose of 0.05 mg. of testosterone propionate when it is administered in 0.05 cc. of oil. When given in 0.50 cc. of oil the reaction is weaker in the ventral prostate and almost imperceptible in the seminal vesicle.

4. At 46 hours after injection, the ventral prostate displays mitotic activity in response to 0.013 mg. of testosterone propionate; the seminal vesicle does not and therefore shows the higher threshold of response.

5. Mitosis is somewhat delayed in the ventral prostate of rats castrated only 10 or 20 days when treated with 0.1 mg. of testosterone propionate. The seminal vesicle responds at about the same time in 10-, 20- and 40-day castrated animals.

6. Mitotic activity and growth are evident in the seminal vesicle at 55 hours after one injection (100 R. U.) of estradiol benzoate. The ventral prostate shows no change from the untreated condition in the castrated rat.

7. Forty milligrams of progesterone, in two daily doses cause hypertrophy in the glandular epithelium and the connective tissue of both the accessory glands 55 hours after the first injection. Mitotic cells are much more numerous in the ventral prostate than in the seminal vesicle.

8. The intensity of the mitotic activity and the simultaneous response of the ventral prostate and the seminal vesicle to doses as small as 0.1 mg. is specific for testosterone propionate.

9. Mitosis in the accessory sex glands, modified by colchicine, is suggested as a sensitive test for androgens.

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PLATE 1

EXPLANATION OF FIGURES

(All figures $\times 536$)

4 Seminal vesicle of an 81-day-old rat which had been castrated on its fortieth day of age and was colchicine-treated 6 hours before death.

5 Seminal vesicle of rat castrated on day 40, injected on day 80 with 0.1 mg. of testosterone propionate, and sacrificed 47 hours later; it was injected with colchicine 6 hours before death.

6 Ventral prostate of rat castrated on day 40, injected on day 80 with 0.1 mg. of testosterone propionate, and sacrificed 47 hours later without colchicine treatment.

7 Ventral prostate of the same rat as figure 5. Note the occurrence of a prophase nucleus (p) in the colchicine treated gland.

8 Ventral prostate of an 82-day-old rat which had been castrated on day 40; it received colchicine 6 hours before sacrifice.

9 Ventral prostate of a rat which had been castrated on day 40, was injected with 0.013 mg. of testosterone propionate on day 80, and was sacrificed 46 hours later after 6 hours of colchicine treatment.

